Ultrasensitive detection of PrP\textsuperscript{Sc} in the cerebrospinal fluid and blood of macaques infected with bovine spongiform encephalopathy prion

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Prion diseases are characterized by the prominent accumulation of the misfolded form of a normal cellular protein (PrP\textsuperscript{Sc}) in the central nervous system. The pathological features and biochemical properties of PrP\textsuperscript{Sc} in macaque monkeys infected with the bovine spongiform encephalopathy (BSE) prion have been found to be similar to those of human subjects with variant Creutzfeldt–Jakob disease (vCJD). Non-human primate models are thus ideally suited for performing valid diagnostic tests and determining the efficacy of potential therapeutic agents. In the current study, we developed a highly efficient method for \textit{in vitro} amplification of cynomolgus macaque BSE PrP\textsuperscript{Sc}. This method involves amplifying PrP\textsuperscript{Sc} by protein misfolding cyclic amplification (PMCA) using mouse brain homogenate as a PrP\textsuperscript{C} substrate in the presence of sulfated dextran compounds. This method is capable of amplifying very small amounts of PrP\textsuperscript{Sc} contained in the cerebrospinal fluid (CSF) and white blood cells (WBCs), as well as in the peripheral tissues of macaques that have been intracerebrally inoculated with the BSE prion. After clinical signs of the disease appeared in three macaques, we detected PrP\textsuperscript{Sc} in the CSF by serial PMCA, and the CSF levels of PrP\textsuperscript{Sc} tended to increase with disease progression. In addition, PrP\textsuperscript{Sc} was detectable in WBCs at the clinical phases of the disease in two of the three macaques. Thus, our highly sensitive, novel method may be useful for furthering the understanding of the tissue distribution of PrP\textsuperscript{Sc} in non-human primate models of CJD.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), commonly known as prion diseases, are fatal neurodegenerative disorders that affect both animals and humans (Collinge, 2001). Prion diseases are characterized by the prominent accumulation of a misfolded prion protein, PrP\textsuperscript{Sc}, in the central nervous system (Prusiner, 1991, 1998). PrP\textsuperscript{Sc}, which is rich in beta-sheet structures and resistant to digestion by proteases and various inactivating treatments (Caughey et al., 1991; Pan et al., 1993), is considered to be the infectious agent for TSEs and appears to self-propagate through post-translational modification of the normal prion protein PrP\textsuperscript{C} (Prusiner, 1998).

One type of human prion disease, Creutzfeldt–Jakob disease (CJD), can be aetiologically identified as sporadic, inherited or acquired by infection (Ironside, 1998; Belay, 1999; Glatzel et al., 2002; Geissen et al., 2007). In variant CJD (vCJD), which is a form of CJD caused by consumption of foods contaminated with bovine spongiform encephalopathy (BSE) prions (Will et al., 1996; Hill et al., 1997; Ironside, 2010), small amounts of PrP\textsuperscript{Sc} have been found in a broad range of peripheral tissues, including the lymph nodes, tonsils, spleen, kidneys, portions of the intestinal tract and skeletal muscle (Wadsworth et al., 2001; Hilton et al., 2004; Peden et al., 2006; Notari et al., 2010), as well as in the
central nervous system. These observations have led to serious concerns that the disease could spread in humans via blood transfusions (Wroe et al., 2006; Knight, 2010) and through the use of contaminated biological and surgical instruments. In order to effectively prevent the spread of this disease, it is important to be able to detect PrPSc as soon after infection as possible, and then, it is crucial to avoid PrPSc contamination in human-derived materials. As the concentration of PrPSc in the tissues or body fluids of infected subjects is predicted to be extremely low until marked clinical signs appear, development of both a sensitive method for detecting PrPSc and animal models to confirm its validity are necessary.

Several studies have used non-human primates to study the transmissibility of human prion diseases (Gajdusek et al., 1968; Gibbs et al., 1968), and the transmissibility of BSE has specifically been investigated using macaque monkeys (Lasmézas et al., 1996, 2005; Comoy et al., 2008; Ono et al., 2011a, b). These studies have reported a number of advantages of using non-human primate models of prion disease. For example, the pathological feature of florid plaques in the brain tissue of BSE-infected macaques and the biochemical characteristics of the PrPSc glycoform profile in these macaques have been shown to be identical to those in human subjects with vCJD (Lasmézas et al., 1996). In macaques inoculated with the BSE prion either intracerebrally or orally and in humans infected with vCJD, PrPSc has been found to be distributed in various peripheral tissues, such as the lymph nodes, spleen, tonsils and muscles. These findings strongly support the possibility that vCJD is caused by an exogenous infection of a BSE prion. Furthermore, BSE can be transmitted via intravenous inoculation (Lasmézas et al., 2001), indicating that macaques can serve as model animals for suspected cases of secondary transmission (via blood transfusion) of vCJD in humans. Therefore, non-human primate models are ideally suited for assessing methods for diagnosis and treatment of prion diseases.

In scrapie-infected rodents (Brown et al., 1998) and sheep (Houston et al., 2008) as well as in deer with chronic wasting disease (CWD), bodily fluids such as the blood, urine, saliva and faeces have been reported to be infectious (Mathiason et al., 2006; Haley et al., 2009b; Mathiason et al., 2010). By using the protein misfolding cyclic amplification (PMCA) technique, which amplifies PrPSc in vitro using normal brain homogenates as the PrPSc substrate, PrPSc has been detected in a variety of bodily fluids, including the blood, cerebrospinal fluid (CSF), urine, faeces, saliva and milk of prion-infected animals (Saborio et al., 2001; Sáa et al., 2006; Murayama et al., 2007, 2010; Thorne & Terry, 2008; Terry et al., 2009; Maddison et al., 2009, 2010; Haley et al., 2009a, 2011; Tattum et al., 2010; Gough et al., 2012). Furthermore, several reports have described the successful detection of PrPSc in bodily fluids of humans with CJD (Orrú et al., 2009; Atarashi et al., 2011; Edgeworth et al., 2011; Peden et al., 2012; Rubenstein & Chang, 2013). For example, PrPSc in the CSF of patients with sporadic CJD (sCJD) and vCJD has been detected using the quaking-induced conversion technique (Atarashi et al., 2007), which detects PrPSc-triggered formation of amyloid fibrils of recombinant prion proteins. Similarly, PrPSc has been detected in the CSF of patients with sCJD using PMCA followed by a sensitive immunoassay termed SOFIA (Rubenstein & Chang, 2013), and bead-captured ELISA has been used to detect blood PrPSc in patients with vCJD (Edgeworth et al., 2011). Therefore, bodily fluids may have high utility as diagnostic materials for CJD. However, the quantitative changes of PrPSc in bodily fluids of non-human primate models of CJD has not yet been determined due to a lack of sensitive methods for assessing very small amounts of prions in these animal models.

In the present study, we have developed a highly efficient PMCA method suitable for cynomolgus macaque BSE PrPSc amplification. This method, which involves amplifying PrPSc using xenogeneic (mouse) PrPC substrate in the presence of sulfated dextran compounds, is capable of amplifying a very small amount of PrPSc from the CSF, blood, and peripheral tissue of BSE-infected macaques. We further investigated CSF and blood PrPSc levels during the period from the latent to terminal stages of the disease and compared PrPSc dynamics in macaques.

**RESULTS**

**Amplification of cynomolgus macaque BSE PrPSc by PMCA**

We first examined the amplification efficiency of PMCA, using the brain homogenate of BSE-infected cynomolgus macaque no. 7 as the PrPSc seed. Before amplification, distinct signals of protease-resistant PrP (PrPres) were detected in brain homogenates diluted up to 10−2 by Western blot analysis (Fig. 1a). In the absence of potassium dextran sulfate (DSP), brain homogenates derived from the squirrel monkey and cynomolgus macaque were not suitable for amplification of cynomolgus PrPSc (Fig. 1b, upper panel). Similarly, no significant amplification was observed using cow, TgBoPrP and PrP0185 mice (Fig. 1b, middle panel), or hamster brain homogenates (Fig. 1b, lower panel) as PrPSc substrates. On the other hand, amplification of PrPSc was achieved in samples diluted to 10−3 and 10−4 when the WT mouse brain homogenate was used as the PrPSc substrate (Fig. 1b, lower panel). Furthermore, amplification efficiency of mouse PrPSc for PMCA was significantly improved in the presence of DSP, and PrPres signals were detected in samples diluted to 10−5 after one round of amplification. On the other hand, DSP was less effective in increasing signal intensity of PrPres after amplifications derived using squirrel monkey, cynomolgus macaque, cow, TgBoPrP mouse and hamster brain homogenates. The detection sensitivity for cynomolgus PrPSc for these PCMs was lower than for PMCA conducted using WT mouse brain homogenate. Higher background signal in the no-seed samples was observed after amplification was conducted using macaque brain homogenate in the presence of DSP.
Detection sensitivity of cynomolgus macaque BSE PrPSc

PMCA using WT mouse brain homogenate containing DSP as the PrPSc substrate was used for amplification of cynomolgus macaque PrPSc. On the basis of our preliminary experiments, the optimal concentration of DSP was estimated to be 1% (w/v); therefore, we used 1% (w/v) DSP for subsequent experiments. We determined the detection limit of the interspecies PMCA technique and confirmed that PrPSc present in a 10⁻⁵ dilution of infected brain homogenate could be detected after one round of amplification, and both 10⁻⁶ and 10⁻⁷ dilutions were positive for PrPSc after two rounds of amplification (Fig. 2a). After three rounds of amplification, PrPrew signals were detected in the samples diluted to 10⁻⁴ and 10⁻³. A PrPrew signal was detected in the 10⁻¹0 dilution samples after four rounds of amplification, but almost no signal was detected in the more extreme dilutions, even after seven rounds of amplification. Thus, compared with no amplification, amplification improved the PrPSc detection sensitivity by a factor of 10⁸. No typical PrPrew signal was detected in samples that contained normal brain homogenate diluted 1 : 10 with mouse PrPSc substrate (Fig. 2b). In addition, the generation of spontaneous PrPrew, as has been reported for amplification in the presence of polyanions (Deleault et al., 2007; Wang et al., 2010), was not observed in 16 samples that contained only mouse PrPSc substrate following seven rounds of amplification (Fig. 2c).

Fig. 1. Amplification of macaque PrPSc using normal brain homogenates derived from several animal species as PrPSc substrates. (a) Ten per cent brain homogenate of BSE-affected cynomolgus macaque was diluted to 10⁻¹ to 10⁻³ in a normal macaque brain homogenate, an undiluted sample (0) was also included. The diluted samples were analysed by Western blot after digestion with protease K (PK). (b) PrPSc seed (10% brain homogenate of BSE-affected cynomolgus macaque) was diluted to 10⁻³ to 10⁻⁵ in normal brain homogenates obtained from the squirrel monkey, cynomolgus macaque, cow, mixture of TgBoPrP and PrP0/0 (TgBoPrP + PrP0/0) mice, mouse and hamster. The diluted samples were amplified in the presence (+) or absence (−) of 1% (w/v) DSP. After amplification, the samples were digested with PK and analysed by Western blot. ‘N’ denotes unseeded control samples in which normal brain homogenate that did not receive a PrPSc seed were processed and analysed in the same manner as PrPSc-seeded samples. The molecular masses of marker proteins are indicated (kDa).
PrPSc distribution in the peripheral tissues of BSE-affected macaques

We examined PrPSc distribution in macaques that were intracerebrally administered a brain homogenate prepared from a BSE-infected cow. In BSE-infected macaques, PrPSc was detected by conventional Western blot analysis in several peripheral nervous tissues and lymph nodes (Table S1, available in the online Supplementary Material). By using serial PMCA, PrPSc was detected in all examined tissues, including: the peripheral nerves, lymph nodes, spleens, tonsils and adrenal glands (Fig. 3). Most samples were found to be positive for PrPSc after no more than two rounds of amplification. On the other hand, PrPSc was detected after three rounds of amplification in four and two of the quadruplicate samples of the tonsil of macaque no. 10 (Fig. 3b) and spleen of macaque no. 11 (Fig. 3c), respectively. No typical PrPres signal was detected in the peripheral nerves, lymph nodes, ileum and glands of an uninfected control macaque (Fig. S1).

PrPSc levels in the CSF

The amplification results for the CSF samples collected from the three macaques are illustrated in Fig. 4. No typical PrPres signal was observed in samples that contained only

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**Fig. 2.** Detection sensitivity for cynomolgus macaque PrPSc. (a) PrPSc seed was diluted to 10⁻³ to 10⁻¹ with PrPC substrate (10% normal mouse brain homogenate), and the samples were serially amplified in the presence of 1% (w/v) DSP. The amplified samples were analysed after each round of amplification (R1–R7) by Western blot after proteinase K (PK) digestion. (b) Normal brain homogenate was diluted to 10⁻³ with the PrPC substrate (lanes a–p), and the samples were serially amplified in the presence of 1% (w/v) DSP. After amplification, a band with a molecular mass similar to that for PrPSc was occasionally observed, which likely corresponds to a residue of the normal isoform of prion protein resulting from incomplete PK digestion. (c) No spontaneous generation of PrPSc was observed in no-seed samples. Lanes a–p contained only PrPC substrate and were amplified in the presence of 1% (w/v) DSP. Exclusive pipettes, a vortex mixer, and a centrifuge were used for handling unseeded samples. The molecular masses of marker proteins are indicated (kDa). NT, Not tested.
Fig. 3. Tissue distribution of PrP\textsuperscript{Sc} in macaques intracerebrally inoculated with BSE. Tissue distribution of PrP\textsuperscript{Sc} in the terminal disease stage in macaque no. 7 (a), no. 10 (b) and no. 11 (c). Quadruplicate samples of each tissue were serially amplified, and the samples were analysed by Western blot following digestion with proteinase K after each round of amplification (R1–R3). The molecular masses of marker proteins are indicated (kDa). N, Nerve; LN, lymph node; Ns, no-seed samples; NT, not tested.
Fig. 4. The appearance of PrP<sup>Sc</sup> in the cerebrospinal fluid (CSF) of BSE-infected macaques. CSF was collected at several points after intracerebral inoculation. Quadruplicate or duplicate CSF samples from BSE-infected macaque no. 7, no. 10, and no. 11 were analysed by Western blot following digestion with proteinase K after each round of amplification (R1–R7). PrP<sup>Sc</sup> was also evaluated in CSF samples from an uninfected control macaque (no. 8C). Dpi, Days post-inoculation. Dpi written in boldface represents clinical stages of the disease. The molecular masses of marker proteins are indicated (kDa). Ns, No-seed samples; NT, not tested.
mouse PrP<sup>C</sup> substrate (lanes Ns), or samples that contained normal macaque CSF diluted 1:10 with mouse PrP<sup>C</sup> substrate (Fig. 4, no. 8C and Fig. S2). PrP<sub>res</sub> signal was not detected in the samples collected 515–208 (macaque no. 7), 509–208 (macaque no. 10) and 490–133 days (macaque no. 11) before disease onset. The existence of PrP<sup>Sc</sup> in the CSF samples was confirmed after the onset of clinical signs. For example, macaque no. 7 presented with early neurological clinical signs of the disease such as slight tremor, startle response and festinating gait. PrP<sub>res</sub> signal was detected after four rounds of amplification in one of the quadruplicate samples collected at this time [867 days post-inoculation (p.i.)], but no other sample was positive for PrP<sup>Sc</sup> even after seven rounds of amplification. Consistent with disease progression, macaque no. 7 presented with ataxia, paralysis of the extremities and rigidity; PrP<sup>Sc</sup> was detected in all of the quadruplicate samples obtained at 987 days p.i. after five rounds of amplification. The macaque finally developed severe dystasia, and after three rounds of amplification, PrP<sup>Sc</sup> was detected in all of the quadruplicate samples obtained at 1100 days p.i. and at the dissection (1127 days p.i.). These observations suggested that the level of PrP<sup>Sc</sup> tended to increase in the CSF as the disease progressed. Although a similar tendency was observed in other macaques, there were differences in the levels of PrP<sup>Sc</sup> in the CSF. For example, duplicate CSF samples collected upon dissection (1067 days p.i.) became positive for PrP<sup>Sc</sup> after two rounds of amplification in macaque no. 10, which showed the shortest latent period of 828 days. On the other hand, the disease developed after a relatively longer latent period of over 1400 days in macaque no. 11, and PrP<sub>res</sub> signals were detected after four rounds of amplification in both samples collected upon dissection (1769 days p.i.).

**PrP<sup>Sc</sup> levels in the blood**

The results of the amplification of white blood cell (WBC) samples collected at several time points after intracerebral administration are illustrated in Fig. 5. No typical PrP<sub>res</sub> signal was observed in samples that contained only mouse PrP<sup>C</sup> substrate (Fig. 5, lanes Ns), or samples that contained normal macaque WBCs (10<sup>4</sup> cells) (Fig. 5, no. 8C and Fig. S2). Furthermore, we confirmed that the WBC matrix had no inhibitory effect on the amplification of PrP<sup>Sc</sup> by serial PMCA (Fig. S3). In macaque no. 7, one of the quadruplicate samples collected upon dissection (1127 days p.i.) became positive for PrP<sup>Sc</sup> after five rounds of amplification. Similarly, PrP<sub>res</sub> signal was detected in one or both of the duplicate samples of macaque no. 11 collected at 1656 days p.i., and at dissection (1769 days p.i.). However, PrP<sup>Sc</sup> was not detected in the blood of these macaques between the latent and the initial stage of disease onset. In macaque no. 10, PrP<sub>res</sub> signal was not detected in the WBCs obtained during the experimental period (320–1067 days p.i.) even after seven rounds of amplification. With regard to plasma samples, no PrP<sup>Sc</sup> was detected in any of the samples collected during the experimental period (data not shown).

**Infectivity of the PMCA product**

The PMCA product obtained after ten rounds of amplification was diluted 10-fold and inoculated intracerebrally into tga20 mice. The tga20 mice inoculated with the PMCA products derived from the brain or WBC PrP<sup>Sc</sup> seeds died after an average period of 305 or 310 days, respectively (Table 1). PrP<sup>Sc</sup> accumulation in the brains of mice was confirmed by Western blot analysis (data not shown). There was no significant difference between the survival periods of these PMCA product-inoculated mice (t-test, P>0.05). Control mice administered with the product containing only PrP<sup>C</sup> substrate survived more than 478 days. These results indicated that both brain- and WBC-derived PrP<sup>Sc</sup> had seeding activities following the PMCA reactions, and the amplified PrP<sup>Sc</sup> maintained their infectious ability during in vitro xenogeneic amplification.

**DISCUSSION**

In the current study, we developed an ultra-efficient PMCA technique for amplifying PrP<sup>Sc</sup> derived from BSE-infected cynomolgus macaques by using mouse brain homogenates with DSP as a PrP<sup>C</sup> substrate and a polyanion additive, respectively. We first proved the existence of PrP<sup>Sc</sup> in the CSF and blood of BSE-infected macaques by PMCA, and showed that cynomolgus macaque BSE PrP<sup>Sc</sup> and non-macaque PrP<sup>C</sup>, effectively converted mouse PrP<sup>C</sup> to a proteinase K (PK)-resistant form. It is well known that PMCA of several xenogeneic combinations of PrP<sup>Sc</sup> seed and PrP<sup>C</sup> substrate can overcome the species barrier (Kurt et al., 2007, 2011; Green et al., 2008; Castilla et al., 2008; Yoshioka et al., 2011; Murayama et al., 2012; Nemeczek et al., 2013), despite the divergent amino acid sequence of prion proteins. Since the BSE prion was transmissible to ICR (WT) mice (Masuji et al., 2008), the cynomolgus macaque PrP<sup>Sc</sup> generated by the cross-species transmission of BSE prion may retain the original characteristics of BSE PrP<sup>Sc</sup>, including structural compatibility with mouse PrP<sup>C</sup> and DSP dependency in PMCA reactions.

PrP<sup>Sc</sup> is detectable in the tonsil, spleen and lymph nodes in vCJD (Wadsworth et al., 2001) and sCJD patients (Rubenstein & Chang, 2013). In an earlier study, PrP<sup>Sc</sup> was found in the lymphoid tissues, including the lymph nodes, spleens and tonsils of macaques intracerebrally inoculated with BSE PrP<sup>Sc</sup> (Lasmézas et al., 1996), as observed in vCJD-inoculated macaques (Lasmézas et al., 2001). Therefore, once PrP<sup>Sc</sup> accumulates in the brain, it may spread centrifugally from the brain to the peripheral tissues through the autonomic nervous system. However, in our previous study, we failed to detect PrP<sup>Sc</sup> in such lymphoid tissues of the BSE-inoculated macaques by conventional Western blotting, except in the submandibular lymph nodes, deep cervical lymph nodes and inguinal lymph nodes (Ono et al., 2011a; Table S1). In the current study, PMCA analysis revealed that PrP<sup>Sc</sup> was distributed in all lymphoid tissues examined in the BSE-infected macaques.
PrPSc levels in most of the lymphoid tissues were extremely low, because PrPSc could only be detected after two or three rounds of amplification. Therefore, significant PrPSc accumulation in the peripheral non-neuronal tissues might not have occurred in these macaques, and PrPSc levels in most lymphoid tissues might have been below the detection limit of the conventional Western blot technique used herein, even at the terminal stage of the disease.

**Table 1.** Mean incubation time following intracerebral inoculation in tga20 transgenic mice

<table>
<thead>
<tr>
<th>Inoculum (R10 PMCA product)</th>
<th>Transmission rate (total death/total number)</th>
<th>Mean survival time ± SD (days)</th>
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<tbody>
<tr>
<td>Brain seed*</td>
<td>100% (6/6)</td>
<td>305 ± 10</td>
</tr>
<tr>
<td>WBCs seed†</td>
<td>100% (6/6)</td>
<td>310 ± 23</td>
</tr>
<tr>
<td>No seed</td>
<td>0% (0/4)</td>
<td>&gt;478</td>
</tr>
<tr>
<td>10% Brain homogenate from a BSE-infected cow‡</td>
<td>100% (20/20)</td>
<td>495 ± 43</td>
</tr>
</tbody>
</table>

R10, Tenth round.
*The final dilution of the infected brain homogenate (macaque no. 7) in the R10 product was $6.4 \times 10^{-11}$.
†The PMCA product from the tenth round of amplification of PrPSc-positive WBCs (macaque no. 7).
‡Classical BSE (c-BSE) prion was inoculated in tga20 mice for comparison of infectivity.

**Fig. 5.** Appearance of PrPSc in the WBCs of BSE-infected macaques. WBCs were collected at several points after intracerebral inoculation. Quadruplicate or duplicate WBC samples from BSE-infected macaque no. 7, no. 10 and no. 11 were analysed by Western blot following digestion with proteinase K after each round of amplification (R1–R7). PrPSc was also evaluated in WBCs from an uninfected control macaque (no. 8C). Dpi, Days post-inoculation. Dpi written in boldface represents the clinical stages of the disease. The molecular masses of marker proteins are indicated (kDa). Ns, No-seed samples.
The origin of PrPSc in WBCs may be the spleen and other lymphoid organs, as suggested previously (Saá et al., 2006). As in humans, PrPC is constitutively expressed in the WBCs of cynomolgus macaques (Holada et al., 2007); therefore, WBCs of cynomolgus macaques can be deemed carriers or reservoirs of PrPSc. Our finding supports the idea that prion diseases may be transmitted via infected blood in primates, as has been previously seen in scrapie-infected sheep (Houston et al., 2008) and CWD-infected deer (Mathiason et al., 2006). An illustration for the appearance of PrPSc in the CSF and WBCs of intracerebrally infected macaques is shown in Fig. 6. PrPSc was found in the WBCs at clinical stages of the disease in macaques no. 7 and no. 11, but PrPSc was not detected in the WBCs of macaque no. 10 throughout the experimental period. Survival time of the BSE-infected macaques ranged from 1067 days to 1769 days. During the period from the onset of clinical signs to the terminal stage of the disease, PrPSc was detected in the CSF in all three BSE-infected macaques. The highest level of PrPSc in the CSF collected upon dissection was observed in macaque no. 10.

A previous study showed that elevated levels of 14-3-3 proteins, which are widely distributed in eukaryotes and play an important role in various signal transduction systems involved in cell proliferation and division, were observed in the CSF of a simian vCJD model (Yutzy et al., 2007). The increase of PrPSc in the CSF probably reflects the leakage of PrPSc from neuronal cells after cell destruction caused by PrPSc infection. We examined 14-3-3 γ levels in the CSF of the BSE-infected macaques (Fig. S4), and found that the signal intensity of the 14-3-3 γ protein became notable after disease onset (no. 7 and no. 10), or in the latter stages of the disease (no. 11). It is worth noting that the highest levels of the 14-3-3 γ protein were observed in the CSF of macaque no. 10 collected at dissection. Therefore, the disease might have progressed most rapidly after a shorter latent period (829 days) in macaque no. 10 than in macaques no. 7 (867 days) and no. 11 (1439 days). Faster accumulation of PrPSc in the brain may cause acute brain damage and result in death before a significant number of infected WBCs begin circulating in the peripheral blood. Macaques no. 7 and 10 both belonged to a breeding colony introduced from the Philippines, and no. 11 was derived from a Malaysian lineage. Thus, the different degrees of disease progression might be related to genetic factors affecting susceptibility or resistance to prion infection.

**Fig. 6.** Schematic illustration for the appearance of PrPSc in the CSF and WBCs of three BSE-infected macaques. After intracerebral inoculation (I. C.), the presence of PrPSc in CSF and WBCs was examined by serial PMCA during the asymptomatic (dotted line) and clinical stages (solid lines). Dpi, Days post-inoculation. Dpi written in boldface represents the clinical stages of the disease. Positive ratio of duplicate or quadruplicate samples was shown as open circle (0 %), closed quadrant (25 %), closed semicircle (50 %), closed three quadrants (75 %) and closed circle (100 %). NT, Not tested.
More detailed studies are needed to clarify the above possibility.

In conclusion, we have developed a highly sensitive method that enables a detailed and precise examination of the distribution of PrPSc throughout the bodies of BSE-infected macaques. We are now conducting experiments analysing oral transmission of the BSE prion and transmission through blood transfusions from BSE-infected macaques. Using our method, PrPSc could notably be detected in bodily fluids obtained during the latent period of the disease in both primate models. Thus, the method developed in this study may be useful in furthering the understanding of the tissue distribution of PrPSc in non-human primate models of CJD.

**METHODS**

**BSE-infected macaques.** This study on non-human primates was conducted according to the rules for animal care and management of the Tsukuba Primate Research Center (Honjo, 1985) and the guiding principles for animal experiments using non-human primates formulated by the Primate Society of Japan (Primate Society of Japan, 1986). The cynomolgus macaques (Macaca fascicularis) examined in this study originated from the Philippines (no. 7 and 10) or Malaysia (no. 11), and were bred at Tsukuba Primate Research Center of the National Institute of Biomedical Innovation. Transmission experiments were approved by the Animal Care and Use Committee (approval ID: DS18-069R1) and Animal Ethics Biosafety Committee (approval ID: BSL3-R-06.01) of the National Institute of Biomedical Innovation. The brain homogenate (200 μl of a 10% brain homogenate) derived from a classical BSE (c-BSE)-infected 83-month-old Holstein (Iwata et al., 2006) was intracerebrally administered to three male macaques (no. 7, 10 and 11) that were 24–29 months in age (Ono et al., 2011a). The animals were housed in biosafety level three animal rooms, and their clinical status was monitored daily. After 35–59 months, the animals were euthanized by anaesthesia overdose following evidence of progressive neurological dysfunction, after which the animals were dissected. A healthy macaque (no. 8 or 28) was used as an uninfected control in this study.

**Sample preparation.** Peripheral nervous and lymphoid tissues were collected upon dissection and stored in small aliquots at −80 °C. Samples from each tissue were homogenized at 10% (w/v) in PBS. WBCs, plasma and CSF were also collected at several time points after inoculation. The blood samples (1.5 ml) were centrifuged at 1500 g for 15 min and the plasma and buffy coat fractions were recovered. Erythrocytes contaminated in the buffy coat fraction were haemolyzed in distilled water, and the samples were stored at −80 °C until analysis.

**Preparation of PrPSc substrates.** To avoid contamination, normal brain homogenates were prepared in a laboratory in which infected materials had never been handled. Brains of a healthy cynomolgus macaque, squirrel monkey (Saimiri sciureus), cow, PrPSc-overexpressing transgenic [Tg(BoPrP) 4092HOZO/PrPm100], TgBoPrP mouse (Scott et al., 1997), PrP-Knockout (PrP0/0) mouse, WT mouse (ICR), and Syrian hamster were homogenized at a 20% (w/v) concentration in PBS containing a complete protease inhibitor cocktail (Roche Diagnostics). The brain homogenates were stored at −80 °C until further use. For analysis, the homogenates were mixed with an equal volume of the elution buffer (PBS containing 2% Triton X-100, 8 mM EDTA) and incubated at 4 °C for 1 h with continuous agitation. After centrifugation at 4500 g for 5 min, the supernatant was used as the PrPSc substrate. When using brain homogenates of TgBoPrP mice, the supernatants were mixed in a 5:1 proportion of PrP0/0:TgBoPrP, and this mixture was used as the PrPSc substrate.

**PMCA.** For the amplification of brain PrPSc, the BSE-infected brain homogenate of macaque no. 7 was diluted from 10−2 to 10−3 with normal brain homogenates from several animal species in an electron beam-irradiated polystyrene tube (total volume, 100 μl). Amplification was performed in the presence or absence of 1% (w/v) DSP, which has been shown to markedly improve in vitro amplification efficiency of bovine BSE PrPSc (Murayama et al., 2010). Amplification was carried out with a fully automatic cross-ultrasonic protein activating apparatus (Elestein 070-CPR; Elektron Science Corporation), which had the capacity to generate high ultrasonic power (700 W). PMCA was performed by 40 cycles of sonication in which a 3 s pulse oscillation was repeated five times at 1 s intervals, followed by incubation at 37 °C for 1 h with agitation.

To examine the sensitivity of interspecies PMCA using the mouse PrPSc substrate for the detection of macaque BSE PrPSc, the 10% infected brain homogenate was serially diluted from 10−3 to 10−12 with mouse PrPSc substrate containing 1% (w/v) DSP (total volume, 80 μl) in an electron beam-irradiated eight-strip polystyrene tube specially designed for PrPSc propagation (Murayama et al., 2010). To obtain maximum amplification efficiency and reduce non-specific background signal in Western blot analysis, a series of amplification steps were programmed as follows: PMCA was performed with 40 cycles of sonication in which a 15 s oscillation and subsequent incubations at 31 °C for 1 h were repeated 10 times; a 15 s oscillation and subsequent incubations at 33 °C for 1 h were repeated 10 times; an intermittent oscillation (3 s pulse oscillation was repeated five times at 1 s intervals) and subsequent incubations at 35 °C for 1 h were repeated 10 times; and finally intermittent oscillations (3 s pulse oscillation was repeated five times at 1 s intervals) and subsequent incubation at 37 °C for 1 h were repeated 10 times. The amplified product obtained after the first round of amplification was diluted 1:5 with the PrPSc substrate, and a second round of amplification was performed. This process was repeated for a maximum of six times.

For amplifying PrPSc in various tissues from BSE-inoculated macaques, the mouse PrPSc substrate containing 1% (w/v) DSP was mixed with a 1/10 volume of homogenized samples or bodily fluids (total volume 80 μl) in eight-strip polystyrene tubes. The WBC pellet (approx. 105 cells) was dissolved in 8 μl of the elution buffer and used as a seed. Serial PMCA was then performed using the four-step amplification programme as described above.

**Western blotting.** After each round of amplification, samples of 10 μl were mixed with 10 μl of PK solution (100 μg PK ml−1) and incubated at 37 °C for 1 h. The digested materials were mixed with 20 μl of 2× SDS sample buffer and incubated at 100 °C for 5 min. The samples were separated by SDS-PAGE and transferred onto a PVDF membrane (Millipore). After blocking, the membrane was incubated for 1 h with HRP-conjugated T2 mAb (Hayashi et al., 2004; Shimizu et al., 2010) at a 1:10,000 dilution. The T2 antibody, which recognizes a discontinuous epitope in amino acid residues 132–156 in the mouse PrP sequence, also reacts with hamster and monkey PrP. After washing, the blotted membrane was developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore), according to the manufacturer’s instructions. Chemiluminescence signals were analysed with the Light Capture system (ATTO).

**Bioassay.** A 10% brain homogenate from BSE-infected macaque (no. 7) was diluted to 10−3 with WT mouse PrPSc substrate containing 1% (w/v) DSP and amplified. The 1:5 dilution of the PMCA product
and its subsequent amplification was repeated nine times. The product from the tenth round was diluted 1:10 with PBS and inoculated intracerebrally (20 μl per mouse) into tga20 mice (Fischer et al., 1996) that overexpress mouse PrPSc. Infectivity of the PMCA product from the tenth round of amplification of a PrPSc-positive WBC sample from macaque no. 7 obtained at dissection 1127 days p.i. was also examined. The PMCA product from the tenth round of amplification of no-seed sample was inoculated as negative control. In addition to the PMCA products, 10% brain homogenate of a c-BSE infected cow was also inoculated into tga20 mice to compare infectivity. The bioassay experiments were approved by the Animal Care and Use Committee of the National Institute of Animal Health (approval ID: 09-44) and were conducted in accordance with the guidelines for animal transmissible spongiform encephalopathy experiments of the Ministry of Agriculture, Forestry and Fisheries of Japan.

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